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HCV Core Immunodominant Region Analysis Using Mouse Monoclonal Antibodies and Human Sera: Characterization of Major Epitopes Useful for Antigen Detection

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Monoclonal antibodies (MAbs) were generated by immunizing mice with a truncated recombinant protein corresponding to the immunodominant region (residues 1–120) of hepatitis C virus (HCV) nucleocapsid protein. The specific recognition by either human sera or mouse monoclonal antibodies of overlapping peptides spanning the core region 1–120 as well as the comparison with epitopes described earlier allowed the fine mapping of HCV core. Within the region 1–120, the major antigenic domain could be restricted to the first 45 amino acids. Indeed, the peptide S42G (residues 2–45) allowed the detection of an anti-HCV core response by all anticore-positive human sera examined. According to their epitope localization, three groups of mouse MABs could be evidenced that were directed against different regions of core. Group II MAbs recognized a strictly linear epitope (QDVKF, residues 20–24), whereas group I MABs were directed against a conformational epitope mainly located at the amino acid residues (QIVGG, 29–33). The epitope of group III MABs was also conformational (PRGRRQPI, residues 58–65). These three epitopes appeared close but different from the three major human epitopes RKTKRNTN, VYLLPR, and GRTWAQPGYPWPLY (residues 7–17, 34–39, and 73–86, respectively). Group II MAB 7G12A8 and group I MAB 19D9D6 were used in a sandwich ELISA for the capture and the detection, respectively, of viral core antigen in sera of patients with chronic HCV infection. After treatment of sera with triton × 100 in acidic conditions, amounts of viral antigen as low as 20 pg/ml of sera could be detected. ***J. Med. Virol. 56:300–309, 1998.***

KEY WORDS: core peptides; anti-HCV core monoclonal antibodies; immune response; clinical diagnosis

INTRODUCTION

Hepatitis C virus (HCV) is now the main etiological agent of posttransfusion non-A, non-B hepatitis. The viral genome is a positive-stranded RNA of approximately 9.5 kb and has one large open reading frame that encodes for a polyprotein of 3,011 amino acids [Choo et al., 1989]. This precursor is then posttraductionally cleaved into putative structural and nonstructural proteins. Among the structural proteins, the core or nucleocapsid region (amino acids 1–191) has been shown to have a high degree of homology between different strains of the virus [Takeuchi et al. 1990; Houghton et al., 1991] and to elicit an antibody response rapidly after the onset of infection. A marked correlation was found between the detection of IgG anti-HCV core and the presence of serum HCV RNA sequences in chronic hepatitis C patients [Okamoto et al., 1990]. Thus, HCV core is an important target for diagnosis of HCV infection by detecting either specific anticore antibodies or circulating viral antigen [Nakagiri et al., 1995; Park et al., 1995].

The analysis of the antibody response with recombinant core fragments and synthetic peptides has identified a major immunodominant region at the amino terminal end of the protein [Hosein et al., 1991; Nasoff et al., 1991; Kotwal et al., 1992; Okamoto et al., 1992; Sato et al., 1994]. The characterization of human epitopes has been reported [Sällberg et al., 1992; Cerino et

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al., 1993; Siemoneit et al., 1994]. Mouse monoclonal antibodies (MAbs), using the whole 22-kDa core protein as the immunogen, were recently characterized [Ferns et al., 1996; Moradpour et al., 1996] and used as detection reagents [Kashiwakuma et al., 1996; Orito et al., 1996].

In this study, we report the characterization of monoclonal antibodies generated by immunizing mice with a truncated core protein corresponding to the immunodominant domain (1–120) defined by Hosein et al. [1991]. The specific recognition by either human sera or mouse monoclonal antibodies of overlapping peptides spanning the core region 1–120, as well as the comparison with epitopes already described, allowed the fine epitope mapping of the antigenic domain of HCV core, which could be restricted to the first 45 amino acids of the protein. Moreover, two monoclonal antibodies directed against epitopes located within this region were used for the capture and the detection of HCV core antigen. As an attempt toward the development of an ELISA for immunodiagnosis, it was possible to detect viral antigen in sera from patients with chronic HCV infection.

MATERIALS AND METHODS

Human Sera

Serum samples were obtained from Professor C. Trépo, U271 INSERM. All sera were tested by second-generation anti-HCV ELISA (Abbott Laboratories, Abbott Park, IL) and second-generation recombinant immunoblot assay, RIBA (Ortho Diagnostic Systems, Raritan, NJ), to detect HCV anticore antibodies. The epitope mapping study included sera from 34 HCV patients, 5 non-HCV patients, and 6 healthy individuals. Viral antigen detection included 12 HCV RNA-positive sera from hepatitis patients in chronic phase. HCV RNA was measured using the quantified branched DNA (bDNA) assay (Quantiplex HCV-RNA 2.0, Chiron Corporation, Emeryville, CA).

Cloning of HCV Core Recombinant Proteins

HCV RNA from serum samples was extracted and reverse-transcribed as described previously [Li et al., 1992]. Amplification of cDNA representing a part of the core protein coding region (amino acids 1–120) of HCV-1a genotype was carried out by polymerase chain reaction (PCR) using primers corresponding to the 5′ and 3′ end of the core 120 protein gene plus *Bam*H1 and *Eco*R1, sites respectively. PCR products were then cloned into the expression plasmids pGEX-3 (Pharmacia, France) or pET-21b (Novagen, U.K.), both double-digested with restriction enzymes *Bam*H1 and *Eco*R1. The resulting plasmids, pGEx-core and pET-core, encode the HCV core protein fused to a glutathione-S-transferase (GST) protein [Smith and Johnson, 1988] and to a hexahistidine peptide, respectively [Arnold, 1991]. These two fusion proteins were called C120-GST and C120-His, respectively.

Bacterial Expression and Purification of Recombinant Proteins

After transformation of pGEx-core and pET-core plasmids in *Escherichia coli* strain Bl 21(DE3), bacteria were grown overnight at 37°C. One-tenth diluted cultures were further incubated at 37°C for 1 hr and then induced by adding β-D-thiogalactopyranoside (IPTG, GIBCO-BRL, Bethesda, MD) to a final concentration of 0.1 mM and 0.4 mM, respectively. The cultures were incubated for an additional 4 hr and bacteria were harvested by centrifugation. The C120-GST and C120-His proteins were purified onto Gluthatione Sepharose 4B (Pharmacia) and onto metal chelation chromatography (Ni-NTA Resin, Qiagen, France).

The purified protein preparations were characterized by SDS-PAGE and Western blot using a pool of HCV-positive sera and either rabbit anti-GST protein or monoclonal antibody against hexahistidine (Dianova, France).

Monoclonal Antibodies

BALB/c JYco female mice, 4 to 6 weeks old (IFFA Credo, Les Oncins, l'Arbresle, France), were immunized by intraperitoneal injection with 10 μg of purified HCV C120-GST fusion protein, emulsified with an equal volume of Freund's complete adjuvant. Six injections were then undertaken by using incomplete adjuvant every 2 weeks. Four days after the last injection, spleen cells were harvested and fused according to Köhler and Milstein [1975, 1976] with the Sp 2/0-Ag14 mouse myelona cell line. After 12–14 days, the culture supernatants were screened by ELISA, in which the solid phase was coated with the antigen used for immunization. Positive colonies were subcloned twice by limiting dilution.

Ascitic fluids were obtained from mice primed with a 0.5-ml intraperitoneal injection of Pristane and then injected with 10^6 Hybridoma cells. IgG antibodies were purified on a protein A-Sepharose 4FF column according to the instructions of the manufacturer (Pharmacia).

Biotinylation of Monoclonal Antibodies

Purified monoclonal antibodies were biotinylated using Sulfo-NHS-LC-Biotin (Merck, Rockford, IL) according to Gretch et al. [1987].

Peptide Synthesis

Peptides corresponding to the selected regions were synthesized on an Applied Biosystems (Foster City, CA) automatic synthesizer (model 431A) using fluorenylmethoxycarbonyl and *t*-butyl protecting groups and trifluoroacetic acid (TFA) deprotection.

The peptides were then purified by reverse-phase high-pressure liquid chromatography (HPLC) using an acetonitrile gradient containing 0.1% TFA as eluant. Composition of the purified peptides was confirmed by amino acid analysis and mass spectrometry.

Peptide Synthesis on Membrane

The simultaneous synthesis of different peptide sequences was carried out on a nitrocellulose membrane utilizing Fmoc amino acid chemistry [Frank and Döring, 1988] (SPOTs Synthesis, Cambridge Research Biochemicals, Cambridge, U.K.). The peptides were synthesized on the support matrix by dispensing small volumes of activated amino acids to spots, which contained free activated amino functions for coupling. During the synthesis, individual coupling reactions could be followed by the color change of bromophenol blue, indicating the disappearance of free amino groups. After washing, residual amino functions were blocked and subsequently the Fmoc-protecting groups were removed, generating free amino functions in the next cycle. Finally, the peptides were acetylated at the amino terminal and side-chain deprotected. By this method, 96 different peptides could be generated on a sheet of paper in the size of a microtiter plate, each in nmole quantities suitable for immunological detection.

Antibody reactivity to the membrane-bound peptides was analyzed by an indirect colorimetric immunoassay. The membrane was incubated overnight in blocking buffer at room temperature (Cambridge Research Biochemicals). After washing once in 50-mM Tris-HCl, pH 8, containing 150-mM NaCl and 0.1% Tween 20, the membrane was incubated with monoclonal antibodies diluted in blocking buffer. The incubation was completed after 3 hr at room temperature. Bound antibodies were detected by incubation with galactosidase conjugated antimouse IgG (Cambridge Research Biochemicals) diluted in blocking buffer.

When developed with appropriate substrate and chromogen (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside and potassium ferricyanide), spots corresponding to peptides with antibody reactivity produce a positive blue signal. Intensity of spots was estimated by visualization and expressed as relative intensity on a scale ranging from 0 to 5.

Indirect ELISA

Ninety-six-well-plate Maxisorb (Nunc, Denmark) were coated with 100-μl/well of peptide or recombinant protein at the concentration of 10 μg/ml in 0.1-M carbonate buffer, pH 8.3. After 2 hr of incubation at 37°C, the plates were washed three times with PBS containing 0.05% Tween-20 (PBS-T) and blocked for 1 hr at 37°C with PBS containing 10% goat serum. Following a second wash with PBS-T, 100 μl of monoclonal antibody diluted in PBS-T containing 10% goat serum were added and incubated for 2 hr at 37°C. After a new wash with PBS-T, the secondary antibody, peroxidase-conjugated goat antimouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) was then added at a 1:5,000 dilution in PBS-T-Goat serum. The plates were incubated for 1 hr at 37°C and then washed once more with PBS-T. The plates were developed using the commercialized bioMérieux color kit containing ortho phenylene diamine and hydrogen peroxyde. After 10 min of incubation, the reaction was stopped with 1.8-N H_2SO_4 and the plates were read at 492 nm with an ELISA plate reader. The values are expressed as the mean OD of triplicate.

ELISA Competition of Monoclonal Antibodies With Themselves and Anticore Human Sera

The plates were coated with C120-GST, blocked and washed as described above. One hundred microliters of the first antibody diluted in PBS-T containing 10% goat serum (MAb at the final concentration of 2 μM or anticore human sera diluted to 1:100) were then added and incubated overnight at 4°C. After three washings, the second MAb that has been previously biotinylated was added at the final concentration of 10 nM and incubated for 1 hr at 37°C. After a new wash with PBS-T, peroxidase-conjugated avidine (Jackson ImmunoResearch Laboratories) was added at a 1:5,000 dilution in PBS-T-Goat serum. The plates were incubated for 1 hr at 37°C and then washed once more with PBS-Tween. The plates were developed and were read as described above. The values are expressed as the mean OD of triplicate.

ELISA Competition of Anticore Human Sera With Monoclonal Antibodies

The plates were coated with C120-GST, blocked and washed as described above. One hundred microliters of MAb diluted at the final concentration of 2 μM, in PBS-tween containing 10% goat serum, were then added and incubated overnight at 4°C. After three washings, a pool of anticore human sera (1:1,000 dilution) was added and incubated for 1 hr at 37°C. After a new wash with PBS-T, peroxidase-conjugated goat antihuman IgG (Jackson ImmunoResearch Laboratories) was added at a 1:5,000 dilution in PBS-T-Goat serum. The plates were incubated for 1 hr at 37°C and then washed once more with PBS-T. The plates were developed and were read as described above. The values are expressed as the mean OD of triplicate.

Detection of C120-His Recombinant Protein

The recombinant core protein was measured by sandwich ELISA. The first monoclonal antibody was coated on solid phase overnight at 4°C, at the final concentration of 5 μg/ml in PBS. The plates were washed three times with PBS-T and then blocked for 1 hr at 37°C with PBS containing 10% goat serum. Following a second wash with PBS-T, 100 μl of C120-His dilutions, treated as described below, were added and incubated for 2 hr at 37°C. After three washings, the second Mab, which has been previously biotinylated, was added at the final concentration of 500 ng/ml and incubated for 1 hr at 37°C. After a new wash with PBS-T, peroxidase-conjugated avidine (Jackson ImmunoResearch Laboratories) was then added at a 1:5,000 dilution in PBS-T-Goat serum. The plates were incubated for 1 hr at 37°C and then washed once more with PBS-T. The plates were developed and were read as described above. The values are expressed as the mean

TABLE I. Immunoreactivity of Anticore Human Sera Against Overlapping Synthetic Peptides Encompassing the Region 2–45[a]

Serum number	Serum dilutions	S42G (2–45)	S18D (2–21)	V22G (25–45)	K22Y (12–35)
1	1/100	>2.5	0.012	0.426	0.435
2	1/100	>2.5	0.02	0.29	0.666
3	1/100	>2.5	0.162	0.503	1.03
5	1/100	>2.5	>2.5	>2.5	>2.5
	1/10000	2.093	0.4	0.578	0.394
6	1/100	>2.5	>2.5	>2.5	>2.5
	1/10000	1.916	0.165	0.237	1.487
7	1/100	>2.5	>2.5	2.241	>2.5
	1/10000	1.565	0.681	0.049	0.335
13	1/100	>2.5	0.297	1.832	1.93
32	1/100	>2.5	0.015	0.579	0.734
33	1/100	0.331	0	0.005	0.31
34	1/100	>2.5	0.483	2.5	1.805
Cut off		0.275	0.045	0.028	0.066

[a]The immunoreactivity of anticore human sera against synthetic peptides was determined by ELISA, as described in Materials and Methods, with sera diluted as indicated. The results are expressed as the mean OD of triplicate. A cutoff of positivity was determined for each peptide. It corresponds to the mean of values obtained by 11 negative sera diluted to 1:100 + 2 standard deviations.

OD of triplicate with C120-His dilutions – the mean OD of triplicate with normal serum alone. The cutoff of detection was calculated with three standard deviations of the normal serum triplicate mean OD. It corresponds to 0.5 pg of protein. Intra- and interassay CV are 9% and 3%, respectively.

Treatments of C120-His Dilutions

C120-His was diluted in normal serum and treated as follows for 45 min at room temperature before being added to ELISA wells: (a) 175 μl of the dilution were incubated with 225 μl of PBS alone; (b) 175 μl were incubated with 45 μl of PBS and 150 μl of 0.1-M sodium acetate, pH 4, containing 0.5-M NaCl; C120-His dilutions were then neutralized with 30 μl of 1-M Tris, pH 8; (c) 175 μl were incubated with 45 μl of 8.5% triton × 100 and 150 μl of 0.1-M sodium acetate, pH 4 containing 0.5-M NaCl; C120-His dilutions were then neutralized with 30 μl of 1-M Tris, pH 8; and (d) 175 μl were incubated with 40 μl of 1% SDS and 185 μl of PBS.

HCV Core Detection in Viremic Sera

HCV core detection was carried out as described above with the couple of MAbs 7G12A8/19D9D6. Viremic sera were treated with triton × 100 and sodium acetate as described for C120-HIS treatment. Amounts of viral core protein were quantified from a comparison with a standard curve obtained using various amounts (0.5 pg–5 ng) of purified C120-His diluted in normal serum and treated in the same conditions as serum samples.

The presence of anti-S42G and anti-S18D antibodies in viremic sera was detected by indirect ELISA as described above with either S42G or S18D as peptide-coated solid phase. Sera were diluted to 1:50.

RESULTS

Immunoreactivity of Anticore Human Sera Against Synthetic Peptides

The immunodominant region of core has been previously located within the 120 N-terminus amino acids of the protein [Hosein et al., 1991]. Thus, the immunoreactivity of 31 human-positive anti-HCV core sera was tested first with three large overlapping synthetic peptides covering the region 2–115: S42G, P42Y, and R40R spanning residues 2–45, 38–82, and 74–115, respectively. A cutoff of recognition was calculated for each peptide (mean of the values obtained with 11 human-negative anti-HCV core sera plus two standard deviations). This showed that the immunoreactivity of sera was predominantly directed against peptide S42G. Indeed, this peptide was recognized by all 31 tested sera, whereas P42Y and R40R were respectively recognized by 23 and 7 out of 31 sera.

The immunoreactivity of positive sera against S42G was then analyzed with three shorter overlapping peptides: S18D, K22Y, and V22G spanning residues 2–21, 12–35, and 22–45, respectively. As shown in Table I, the immunoreactivity of sera against these three peptides indicated the presence of at least one epitope in the sequence of each peptide. However, the same signal of positivity as S42G could be obtained neither with S18D, K22Y, and V22G separately nor by using an accumulation of the three peptides' values. These results suggest the recognition by sera of conformation-dependent epitopes present only in S42G.

Characterization of Monoclonal Antibodies

Nine hybridomas secreting MAbs specific for HCV recombinant core protein C120-GST were selected and their immunoreactivity was tested with peptides previously described. As shown in Table II, these different MAbs could be classified in three groups. Seven MAbs reacted with peptide S42G (sequence 2–45), whereas the last two MAbs reacted with peptide P42Y (sequence 38–82). Four out of the seven MAbs reacting with S42G also reacted with the shorter peptide V22G (sequence 22–45); the other three did not react with either V22G or S18D (sequence 2–21) but reacted with K22Y (sequence 12–35).

TABLE II. Immunoreactivity of Anticore MAbs Against Synthetic Peptides Encompassing the Region 2115[a]

MAbs	Groups	S42G (2–45)	V22G (25–45)	S18D (2–21)	K22Y (12–35)	P42Y (38–86)
19D9D6	I	+	+	–	–	–
14C12F5	I	+	+	–	–	–
27D5G5	I	+	+	–	–	–
1G6C12	I	+	+	–	–	–
7H2D4	II	+	–	–	+	–
7G12A8	II	+	–	–	+	–
2C6F6	II	+	–	–	+	–
2G9A7	III	–	–	–	–	+
2H12H9	III	–	–	–	–	+

[a]The immunoreactivity of anticore MAbs against synthetic peptides was determined by ELISA, as described in Materials and Methods. MAbs were diluted to final concentrations ranging from 20 nM to 100 nM.

TABLE III. Competition of MAbs With Themselves and Anticore Human Sera[a]

	(c) H. Sera	(d) 19D9D6	(d) 14C12F5	(d) 27D5G5	(d) 1G6C12	(d) 7H2D4	(d) 7G12A8	(d) 2C6F6	(d) 2G9A7	(d) 2H12H9
(a) H. sera	nd	44	59	30	13	ND	ND	ND	ND	ND
(b) 19D9D6	19	86	87	70	70	ND	ND	ND	ND	ND
(b) 14C12F5	30	86	87	70	70	ND	ND	ND	ND	ND
(b) 27D5G5	32	91	96	88	84	ND	ND	ND	ND	ND
(b) 1G6C12	69	53	63	52	95	ND	ND	ND	ND	ND
(b) 7H2D4	29	ND	ND	ND	ND	84	94	75	ND	ND
(b) 7G12A8	39	ND	ND	ND	ND	81	92	71	ND	ND
(b) 2C6F6	51	ND	ND	ND	ND	86	95	78	ND	ND
(b) 2G9A7	64	ND	ND	ND	ND	ND	ND	ND	94	90
(b) 2H12H9	47	ND	ND	ND	ND	ND	ND	ND	92	96

[a]ELISA competition experiments were performed as described in Materials and Methods. Results are expressed as inhibition percentage of binding by either human sera (a) or biotinylated MAbs (b), measured under identical conditions but in the absence of preincubation of either human sera (c) or MAB (d) as first antibody. ND: not determined.

Competition of Monoclonal Antibodies With Themselves and Anticore Human Sera

Group I, group II, and group III MAbs were able to inhibit themselves from binding to C120-GST–coated solid phase within their own group (Table III). In contrast, they were partially blocked from binding by a pool of 10 human anti-HCV core-positive sera. The binding of the same pool of human sera to S42G-coated solid phase was also partially inhibited by any of the four group I MAbs.

Epitope Mapping of Monoclonal Antibodies

To identify the epitope recognized by the group I MAbs, overlapping octapeptides offset by one, covering the peptide V22G and spanning residues 16–51 of core protein, were synthesized on nitrocellulose membrane. The four group I MAbs reacted strongly with peptides spanning residues 26–38 and more weakly with peptides corresponding to residues 41–49 (Fig. 1A). The strongest reactivity was obtained with octapeptide QIVGGVYL and the deduced minimal sequence for binding was QIVGG (residues 29–33). However, amino acids contained in peptides corresponding to residues 41–49 as well as 22–28 and 17–25 could participate to the antigen-antibody interaction.

As shown on Figure 1B, the three MAbs of group II reacted with just five overlapping peptides, suggesting that the strongest binding was obtained with the peptide RPQDVKFP (residues 18–25). QDVKF (residues 20–24) was deduced as the minimal motif to obtain IgG binding. According to these results, the epitope recognized by group II MAbs appeared to be strictly linear.

Epitope mapping of the group III MAbs was carried out with overlapping octapeptides covering the P42Y peptide sequence (residues 38–86 of core protein). As shown in Figure 2A, the MAbs reacted most strongly with PRGRRQPI (residues 58–65). However, several peptides corresponding to other residues of P42Y were also significantly recognized by group III MAbs, suggesting that this epitope could be conformational.

Epitope Mapping of Human Antibody Response

In order to study the recognition by anti-HCV core human sera of the epitopes characterized with mouse monoclonal antibodies, the immunoreactivity of a pool of anti-HCV core-positive sera was also tested with overlapping peptides. The binding of human IgG was tested first with peptides spanning core residues 2–21. The immunoreactivity was found with peptides covering residues 7–17, corresponding to the epitope RKTKRNTN previously described [Sällberg et al., 1992].

The binding of human IgG was then tested with peptides spanning core residues 16–51 (Fig. 1C). The

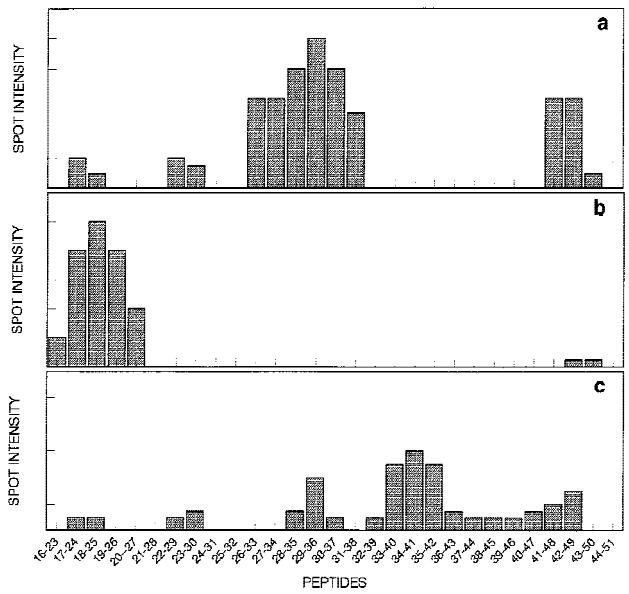

Fig. 1. Immunoreactivity of overlapping octapeptides offset by one, spanning residues 16–51 of core protein. Peptides are indicated by their amino acid locations. **A:** Reactivity of peptides with group I MAbs diluted at the final concentration of 90 nM. **B:** Reactivity of peptides with group II MABs diluted at the final concentration of 66 nM. **C:** Reactivity of peptides with anticore human sera diluted to 1:50. The positive blue signal corresponding to antibody reactivity against peptides synthesized onto spots is expressed as relative intensity on a scale ranging from 0 to 5.

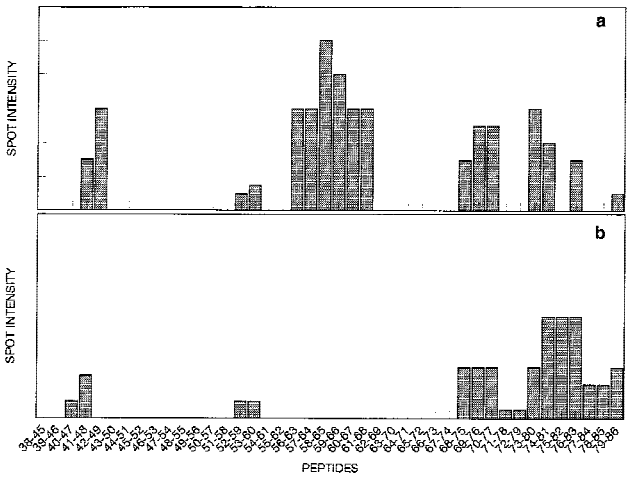

Fig. 2. Immunoreactivity of overlapping octapeptides offset by one, spanning residues 38–86 of core protein. Peptides are indicated by their amino acid locations. **A:** Reactivity of peptides with group III MAbs diluted at the final concentration of 66 nM. **B:** Reactivity of peptides with anticore human sera diluted to 1:50. The positive blue signal corresponding to antibody reactivity against peptides synthesized onto spots is expressed as relative intensity on a scale ranging from 0 to 5.

strongest immunoreactivity was obtained with peptide VYLLPRRG and the minimal sequence for binding appeared to be VYLLPR. Thus, as already suggested by the partial inhibition of group I MAbs binding to S42G in the presence of human sera, the epitope recognized by human antibodies appeared to be close but different from the epitope recognized by mouse monoclonal antibodies. In order to further analyze this result, the QIVGGVYL and VYLLPRRG peptides were again synthesized on nitrocellose membrane and tested with each of five sera. VYLLPRRG was strongly recognized by all five sera with reactivity ranging from 2.5 to 5, while three out of five sera reacted weakly to QIVGGVYL (reactivity ranging from 0.5 to 2). Thus, VYLLPR corresponds to the immunodominant human epitope as previously reported [Cerino et al., 1993; Siemoneit et al., 1994] and QIVGG appears to be a minor human epitope. However, human antibody reactivity was not significantly obtained with peptides corresponding to the linear epitope recognized by mouse group II MABs (Fig. 1C).

The binding of IgG from the same pool of HCV-positive sera was also tested with overlapping octapeptides spanning core residues 38–86. As shown on Figure 2B, the immunoreactivity of human sera was different from that of mouse monoclonal antibodies since the strongest human antibody binding was obtained with peptides spanning residues 73–86, whereas mouse antibodies bound to peptides spanning residues 56–68. This human epitope could also be conformational.

Antigen Detection in Human Sera

An assay based on the capture and detection of HCV core protein by two monoclonal antibodies, directed against two different parts of the protein, was developed in order to quantify the HCV core antigen in human sera. One monoclonal antibody of each group was selected for its high affinity. Group II MAb 7G12A8 and group III MAb 2G9A7 were tested for capturing antigen, and group I MAb 19D9D6 was chosen for detection. A significant detection of HCV recombinant core protein C120-His was obtained with 5 pg of protein by using both couples of MAbs 7G12A8/19D9D6 and 2G9A7/19D9D6. However, HCV core antigen could not be detected in sera from viremic patients without pretreating sera with dissociating agents in order to release the antigen from immunocomplexes and/or from its association with viral RNA. The effect of different serum pretreatments on the capture and sensibility of the assay was tested with both couples of MAbs. Treatments did not affect significantly the detection of core recombinant protein by the couple 7G12A8/19D9D6, although the sensitivity decreased (Fig. 3). However, in the same conditions, the detection of this antigen by the couple 2G9A7/19D9D6 was abolished. Thus, the treatment of viremic sera with Triton × 100 in acidic conditions allowed the detection of HCV core antigen by the couple 7G12A8/19D9D6, in 5 out of 12 sera from individuals with chronic hepatitis in correlation with high amounts of HCV RNA (Table IV).

Among the seven viremic sera in which the core protein was not detected, five sera had lower amounts of HCV RNA, whereas similar amounts of RNA copies (>5.8 and 5.9 × 10^8 copies/ml) were quantified in two sera. Interestingly, when tested for their immunoreactivity against different synthetic peptides, all sera re-

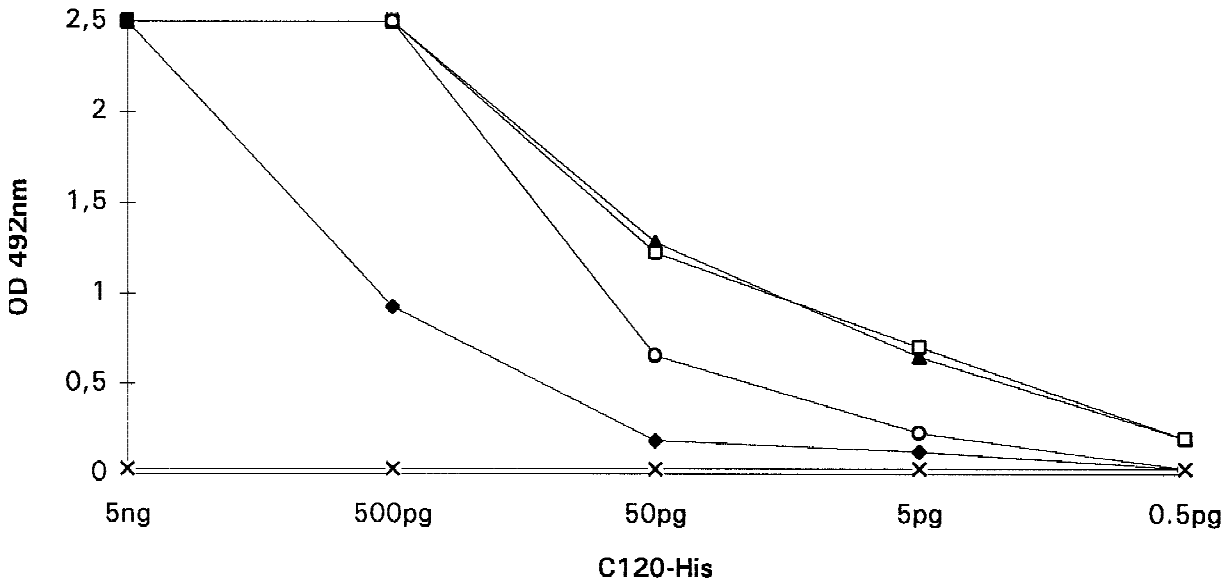

Fig. 3. C120-His detection were performed as described in Materials and Methods with the couple of MAbs 7G12A8/19D9D6. C-120His diluted in normal serum was treated with the following reagents: PBS alone (▲); 0.1-M sodium acetate, pH 4, containing 0.5-M NaCl. C120-His (□); 8.5% triton × 100 and 0.1-M sodium acetate, pH 4, containing 0.5-M NaCl (○); 1% SDS (♦). Limit of detection (×).

TABLE IV. HCV Core Detection in Viremic Sera

Serum number	Core/ml[a]	HCV RNA[b] copies/ml	Anti-S18D Ab[c]	Anti-S42G Ab[c]
35	2.2 ng	$>5.8 \times 10^8$	+	+
36	4 ng	3.1×10^9	–	+
37	2 ng	6.8×10^8	–	+
38	20 pg	6.7×10^7	–	+
39	92 pg	$>5.7 \times 10^8$	+	+

[a]HCV core detection was performed as described in Materials and Methods with the couple of MAbs 7G12A8/19D9D6.
[b]Amounts of HCV RNA quantified in the sera.
[c]The presence of anti-S42G and anti-S18D antibodies in viremic sera was detected in ELISA as described in Materials and Methods. Results are indicated as + or – for presence or the absence of antibody reactivity against peptides S18D and S42G.

act with S42G, but three out the five sera did not react with S18D. These results suggest that, without more powerful treatments, antibodies bound to the NH2 terminus part of the protein could prevent antigen capture by MAb 7G12A8.

DISCUSSION

Analysis of the immunodominant region of HCV core with synthetic peptides showed that antigenic determinants are distributed all along this sequence. However, within this region, the major antigenic domain is contained in the peptide S42G (amino acids 2–45). Indeed, S42G alone allows the detection of an anti-HCV core antibody response since this peptide was recognized by all tested anticore positive human sera. Anticore antibodies were not detected in some patients, although HCV RNA was shown by RT-PCR [Nagasaka et al., 1996]. However, amino acids 1–47 were found to be completely conserved among these patients and a possible defect in the host's immune system was suggested.

Within the S42G sequence, human epitopes could be characterized by testing the reactivity of overlapping octapeptides. The two major linear epitopes previously described and respectively located at residues 7–17 (RKTKRNTN) [Sällberg et al., 1992] and residues 34–39 (VYLLPR) [Cerino et al., 1992; Siemoneit et al., 1994], appeared different from the epitopes recognized by mouse monoclonal antibodies, whereas a minor human epitope detected in only two out five sera at residues 29–33 (QIVGG) coincided with the epitope recognized by group I monoclonal antibodies. However, although RKTKRNTN was contained in peptide S18D, and both VYLLPR and QIVGG were contained in peptide V22G, the same signal of positivity could not be reproduced by the addition of signals obtained with both peptides.

These results suggest the recognition by sera of conformation-dependent epitopes undetected with overlapping octapeptide reactivity. Another possibility is that, for each human epitope characterized, the participation of amino acids only present in the longer peptide S42G stabilized or increased the affinity of the antigen-antibody interaction. Recently, structural analysis and molecular modeling of the nucleocapsid peptide S42G allowed the characterization of a tridimensional motif that is composed of 2 α-helix (R17-F24 and Q29-L37) separated by a loop (P25-G28). The 2 α-helix are maintained perpendicular by hydrophobic interactions [Lavadière et al., 1997; Penin et al., 1997]. This structure could define a conformation-dependent antigenic domain and could thus be critical for optimal immunoreactivity with human sera. It is worth pointing out that the two major human epitopes are located on each side of the loop. Moreover, the epitope recognized by group II MAbs (QDVKF), and which appears to be a new epi-

tope, is close but distinct from the epitope recognized by human antibodies previously described [Cerino et al., 1992; Siemoneit et al., 1994]. This strictly linear epitope (QDVKF) was located at the end of the first α-helix, whereas the epitope recognized by group I MAbs (QIVGG) was located at the beginning of the second α-helix.

A human epitope was also characterized in the region corresponding to the peptide P42Y. This epitope, only recognized by 23 out of 31 sera, was located mainly at residues 73–86. A significant immunoreactivity of human sera was obtained against other octapeptides in the region 38–86, suggesting that this epitope would be conformational. However, significant sequence variations have been identified within amino acid residues 65–81, which may identify two serologically distinguishable core antigen subtypes, corresponding to either genotype I and II or genotype III and IV [Machida et al., 1992]. The reduced sensitivity of antibody reactivity within this region could be due to the absence of recognition of the genotype 1a core protein sequence 65–81 by sera from HCV genotypes III and IV patients. In contrast, the other human epitopes were located to regions with high homologies between the different HCV genotypes [Bukh et al., 1994]. Group III monoclonal antibodies recognition was also directed against a conformational epitope mainly located at residues 58–65 (PRGRRQPI). However, so far no data on the core structure in the region corresponding to P42Y are available.

Generation and characterization of mouse monoclonal antibodies raised against recombinant HCV core protein have not been reported until recently [Ferns et al., 1996; Moradpour et al. 1996]. However, MAbs obtained by Ferns et al. [1996] were all reported as conformational since the Inno-LIA HCV III assay, based on core peptides detection, failed to recognize any of them. Among the three monoclonal antibodies obtained by Moradpour et al. [1996], MAbs C7-50 and C8-59 could recognize an epitope similar or close to the epitope recognized by group I MAbs in the present data, whereas MAb C8-48 was directed against a strain-specific conformational epitope located within the first 82 amino acids. Taken together and compared to the human polyclonal immune response against the native antigen in HCV infection [Sällberg et al., 1994], these results suggest that the immunodominant domain of core elicits a broad range of antibody specificities depending on the immunogen conformation. Indeed, the MAbs characterized in the present study have been raised against the truncated core protein 1–120, the conformation of which could be slightly different from the whole construction as well as the native viral nucleocapsid.

However, the recognition of core epitopes by human and mouse could also be restricted by their respective immune repertoire [Chen et al., 1995; Ahmed et al., 1996]. In addition, differences between mouse and human antibody reactivity against HCV core protein have been reported [Moradpour et al., 1996]. Nevertheless, these mouse MAbs were able to recognize epitopes on the native viral nucleocapsid. Indeed, by using in a sandwich ELISA assay group II MAb 7G12A8 for capture and group I MAb 19D9D6 for detection, the core protein could be detected and quantified in sera from viremic patients. This assay also allowed the detection of this antigen in lysed hepatocytes coming from HCV-infected liver (data not shown). Interestingly, although the epitopes recognized by these two MAbs were very close to each other, no binding inhibition of 19D9D6 by steric hindrance with 7G12A8 was observed. This result could be explained by the conformation of this region [Lavadière et al., 1997; Penin et al., 1997].

The association of hepatitis C virus particles with immunoglobulin [Choo et al., 1995] and very-low-density lipoprotein VLDL [Thomssen et al., 1992; Agnello, 1995] have been reported as an alternative mechanism for persistent infection. Different dissociating treatments proved to be efficient for HIV p24 detection in sera [Nishanian et al., 1990]. Thus, as a first step toward the development of a quantitative antigen assay, the effect of such serum treatments on the sensibility of the assay was studied with recombinant core protein in normal serum. Most treatments abolished the detection of the recombinant protein by the MAb couple 2G9A7/19D9D6 but did not significantly affect its detection by 7G12A8/19D9D6. These results are in agreement with the recognition of a linear epitope by 7G12A8 and a conformational epitope by 2G9A7.

Although MAb 19D9D6 was also directed against a conformational epitope, the strong immunoreactivity of 19D9D6 against the linear sequence QIVGGVYL could explain the ability of this MAb to detect the viral antigen even in dissociating conditions. Moreover, refolding of the captured protein may occur after removing the dissociating agents from the ELISA wells during the washing steps.

Because of the low level of circulating HCV particles and the lack of an easily available cell culture, direct detection of HCV by conventional techniques is very difficult. However, viral proteins are more stable than RNA in serum samples and thus serum levels of HCV core protein may reflect HCV viremia. Assuming that 500 molecules of core protein are associated with each molecule of RNA, Takahashi et al. [1992] have estimated a concentration of about 4×10^7 nucleocapsid particles per ml of plasma. In the present data, taking into account these values, the concentration of nucleocapsid detected in 5 out of 12 viremic sera would range from 2×10^5 to 4×10^7 particles per ml.

However, the quantified HCV RNA in these sera ranged from 6.7×10^7 to 3.1×10^9 RNA copies/ml of serum, indicating that the viral protein could not be detected completely. The amount of viral RNA quantified in the serum of Japanese patients with chronic liver disease was 2×10^5 to 2×10^8 copies/ml [Nakagawa et al., 1994]. Thus, core protein could only be detected in highly viremic sera because of the limit of sensitivity of the assay. But, more likely, antibodies [Takahashi et al., 1992] and/or other bound molecules

could interfere with the capture and detection of core antigen if not separated with an adapted method. Improving serum treatment with dissociating agents and increasing the sensitivity of HCV core detection by the couple 7G12A8/19D9D6 in a fluorescent enzyme immunoassay (FEIA) are currently under development in order to correlate HCV core level with viremia.

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